

# Detection of salmonellae by real-time PCR within 8 hours

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**Abstract:** Rapid detection of salmonella in pork was accomplished by combining immunomagnetic separation (IMS) and real-time PCR. Pork samples were spiked with known numbers of *Salmonella* Senftenberg, homogenized and incubated for 4 hours. IMS was used to detect and concentrate salmonellae out of the homogenised samples. DNA of separated salmonellae was extracted and examined with real-time PCR. Specificity of the applied PCR-system was confirmed with 50 sero-vars of salmonellae and 20 other species of enterobacteriaceae. When the amplicon was stained with SYBR green, the sensitivity of the method was 10 colony forming units (cfu) / g. The application of hybridization probes however lowers the detection limit to 1 cfu / g.

**Keywords:** salmonella diagnostic, fluorescence detection, immunomagnetic separation, LightCycler PCR, hybridization probes

**Introduction:** Even in industrialized countries with high living standards salmonellae are some of the major causes of food poisoning. 85,5 % of food-borne disease outbreaks in Germany from 1993 till 1998, where the agent was identified, salmonellae were the causative agents (7<sup>th</sup> WHO report, 2000). Meat and meat products are important sources of salmonellosis. In a bacteriological examination of samples from slaughtered pigs and the environment 4,4 % of the samples were tested salmonella positiv (v. Altrock et al., 1999). Conventional methods for salmonella diagnosis in food take at least 3 to 5 days (Gardner and Provine, 1987). For preventiv medical reasons this duration is too long. Therefore a method should be developed detecting salmonellae within the same day.

## Materials and Methods:

### Sample preparation

Pork samples of 5g spiked with known numbers of cfus of *Salmonella* Senftenberg and 5ml of buffered peptone water (BPW) are homogenized with an ultra-turrax. 35 ml of BPW are added and the samples are incubated at 37 °C for 4 h.

### Immunomagnetic separation

The probes are centrifugated at 800 rpm for 5 min. 80 µl of magnetic beads coated with anti-salmonella antibodies (Dynabeads®) are added to 10 ml of the supernatant and shaken 10 min with 400 rpm. The beads are recovered by magnetic force and washed 3 times with phosphate buffered saline containing 0,05% of Tween 20.

### DNA extraction:

The salmonella-bead-pellet is resuspended in 30 µl PCR-lysis buffer containing 1 % Proteinase K and incubated for 1 hour at 56 °C and 10 min at 100 °C.

### PCR procedure:

Real-time PCR is performed using the LightCycler System (Roche). 6 µl of the DNA-extract are set in PCR. The selected pair of primers, (forward: TGCCTAC AAGCATGAAATGG; reverse: AAAGTGGACCACGGTGACAA) (Stone et al., 1994) amplifies a sequence of a genus specific region on the *invA* gene of salmonellae (Rahn et al., 1992). The amplification protocol is as follows: denaturation 95 °C for 5 s, annealing 60 °C for 10 s, elongation 72 °C for 18 s, with a MgCl<sub>2</sub>-concentration of 3 mM. The amplicon is monitored with two different detection systems, a DNA double-strand staining fluorescence probe (SYBR Green, Roche) and hybridization probes emitting a signal depending on fluorescence resonance energy transfer. The SYBR Green method is combined with melting curve analysis. The sequence of the hybridization probes is *invA* FL: CGTCTTATCTTG ATTGAAGCCGATGCC and *invA* LC: TGAAATTATCGCCACGTTCTGGGCA (TIB Molbiol, O. Landt).

### Validation of method:

Specificity of the PCR-procedure was tested with 50 different salmonella serovars and 20 other species and strains of enterobacteriaceae (Citrobacter 2 strains, E.coli 4 strains, Edwardsiella 1 strain, Enterobacter 2 strains, Klebsiella 2 strains, Morganella 1 strain, Proteus 3 strains, Serratia 3 strains, Yersinia 2 strains). All species were cultivated on culture and DNA extracted with Proteinase K-PCR-buffer as described above.

The sensitivity of the method, combining preenrichment, IMS and PCR, is demonstrated by detection limits of Salmonella Senftenberg in cfu, monitored with SYBR Green and hybridization probes.

**Results:** All 50 salmonella serovars were detected by both, SYBR Green in combination with melting curve analysis and hybridization probes. Testing of 20 relevant enterobacteriaceae shows more specificity with hybridization probes than with SYBR green. All strains were negative using hybridization probes. With SYBR green 1 strain showed a positive result when running 30 cycles, 4 strains when running 45 cycles.

Applying hybridization probes 1 cfu of Salmonella Senftenberg / g pork was detectable. A tenfold higher concentration was necessary when using SYBR green (fig.1)



Figure 1: Detection limits of Salmonella in pork samples using IMS-PCR

cfu / g pork	SYBR green					hybridization probes				
	1	2	3	4	5	1	2	3	4	5
100000	+	+	+	+	+	+	+	+	+	+
10000	+	+	+	+	+	+	+	+	+	+
1000	+	+	+	+	+	+	+	+	+	+
100	+	negative	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+
1	negative	+	negative	+	negative	+	+	+	+	+

**Discussion:** The described real-time PCR procedure is specific to detect 50 salmonella serovars. However the specificity of hybridization probes is higher than that of SYBR green. The combination of 4 hours preenrichment, IMS and real-time PCR allows to detect salmonellae within 8 hours with a detection limit of 1 cfu / g pork. This rapid method makes early preventiv acting in food hygienics possible.

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